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**(54) Accelerated nucleic acid reassociation method.**

**(57) A method for the formation of double stranded nucleic acid molecules from separate single stranded nucleic acid molecules in a single phase reaction solution is disclosed wherein the rate of reaction is greatly increased over the rate of reaction at standard reference conditions. The greatly accelerated reaction rate is accomplished through the use of known concentrations of nucleic acid precipitating agents which are added to the reaction solution. Nucleic acid denaturing agents may also be added. The solution so formed is incubated and then assayed for the presence of double stranded nucleic acid molecules.**

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ACCELERATED NUCLEIC ACID REASSOCIATION METHOD

BACKGROUND OF THE INVENTION

Field of The Invention

The present invention is directed to a method for the renaturation, reassociation or hybridization of single stranded  
5 nucleic acid molecules into double stranded nucleic acid molecules wherein the rate of reaction is greatly increased over the rate of reaction under standard reference conditions of 0.12M phosphate buffer at 60°C. More particularly, the present invention is directed to a method for the renaturation, reassociation  
10 or hybridization of nucleic acids, including DNA to DNA, RNA to DNA, and RNA to RNA reactions wherein the rate of the reaction is greatly increased by factors of 50 to 100 times, and even up to several thousand times that of the reaction rates observed under standard reference conditions. These greatly accelerated reaction  
15 rates are achieved through the utilization of reaction solutions containing nucleic acid precipitating agents.

Description of The Prior Art

Numerous methods for the nucleation of single stranded nucleic acid molecules into double stranded molecules are known  
20 in the art and have proven to be useful tools for the analysis of genetic material from a wide variety of organisms. Generally speaking, these nucleation reactions, renaturation, reassociation and hybridization, are based on the tendency of single stranded nucleic acid molecules having blocks or segments of complementary  
25 base sequences to form base paired structures between these

complementary sequences and to rewind forming double helices. The greater the extent of sequence complementarity between the single stranded nucleic acid molecules, the greater the tendency for a given pair of molecules to nucleate and form a double  
5 stranded or duplex molecule.

Renaturation, reassociation and hybridization are essentially synonymous reactions between single stranded nucleic acid molecules. As such, they will be discussed interchangeably throughout the body of this paper. However, the following  
10 distinction may prove helpful in understanding the technology involved. Renaturation generally refers to the formation of a double stranded nucleic acid molecule from two single stranded molecules which were originally base paired to one another and separated through a denaturation process. Reassociation refers  
15 to the process of forming double stranded nucleic acid molecules between two single stranded molecules which usually come from different original molecules. Hybridization refers to the formation of double stranded nucleic acid molecules from single stranded nucleic acid molecules of individual origin with respect to  
20 one another. It should be appreciated that these are not clear cut distinctions and that there is considerable overlap between them. For example, DNA:DNA reactions are commonly called both reassociation and hybridization reactions. On the other hand, the formation of an RNA:RNA double stranded molecule is generally  
25 referred to as hybridization.

The kinetics of these reactions are well understood in the art also following second-order kinetics. Thus, as the concentration of the single stranded nucleic acid molecules is increased, the rate of the reaction is also increased. Conversely,  
30 decreasing the concentration of the single stranded nucleic acid reactant will decrease the rate of reaction and thus increase the

time necessary for the formation of the double stranded nucleic acid molecules to take place.

The effect of temperature on the reaction rate is also well known in the art. As the temperature of the reaction decreases below the  $T_m$  (the temperature at which 50% of the double stranded molecules is denatured, also known as the "melting temperature") a maximum rate for the reaction is achieved at temperatures of approximately 15°C to 30°C below the  $T_m$ . Further decreases in temperature are known to decrease the rate below this maximum rate.

Lastly, with respect to the kinetics of these reactions, it is known that the reaction rates are very dependent on the ionic strength below 0.4M for electrolytes such as NaCl and are almost independent of the salt concentration above this ionic strength.

More information on the kinetics and reaction rates of these nucleic acid association reactions can be found in the following publications:

Wetmur, R., and Davidson, N. (1968), J. Molec. Biol. 31, 349;

Wetmur, R. (1975), Biopolymers 14, 2517;

Britten, R., J., Graham, D., and Neufeld, B. (1974), Methods Enzymol. 29, 363;

Kohne, D. E., Levinson, S.A., and Byers, M. J. (1977) Biochemistry 16, 5329; and

Orosz, J. M., and Wetmur, J. G., (1977) Biopolymer 16, 1183.

It has long been recognized in the art that a major limitation on the utility of these known nucleic acid association techniques is the basic rate of the reaction. Reaction times on the order of several hours to tens of hours and even days are

commonplace. Increasing the reaction rate by increasing the quantities of single stranded nucleic acid molecules utilized in the reactions (due to the second-order kinetics) is not a desirable solution to this problem for three reasons. First, in many cases the target single stranded nucleic acid in the reaction is extracted from physiological samples which inherently limits the amount of such nucleic acid available to that contained in the cells of the physiological sample. Secondly, there are significant expenses associated with the use of nucleic acid reactants which limits the practical utility of increasing the quality of reactants. Thirdly, increasing the quantities of single stranded nucleic acid molecules decreases the sensitivity of the reaction by increasing the background noise. Nonetheless, a number of techniques have been developed to increase the basic rate of these reactions by factors of 5 to 50 or more. Techniques of limited applicability have also been developed which increase the basic reaction rate by factors on the order of 1000 or more. However, as will be discussed in detail below, none of these prior art techniques has been successful at producing greatly accelerated reaction rates of 50 to 100 times or more than the basic reference standard reaction in a single phase system applicable to DNA:DNA, DNA:RNA, and RNA:RNA reactions.

When dealing with reaction rates, the accepted standard reference condition for the comparison of these rates is an aqueous solution of 0.12M phosphate buffer (PB) at 60°C. A similar standard reference condition that is often used giving comparable reaction rates is an aqueous solution of 0.18M NaCl at 60°C.

By far the most common technique of accelerating the reaction rate above that of the standard reference condition has been to increase the salt concentration of the reaction solution

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above that of the standard reference condition. As detailed in following table, while significant reaction rate increases are observed by increasing the salt concentration, the prior art techniques indicate that the rate of increase levels off or even falls for salt concentrations above 2M.

TABLE 1

PRIOR ART KNOWLEDGE OF EFFECT OF  
SALT CONCENTRATION ON DNA:DNA HYBRIDIZATION RATES

<u>Salt</u>	<u>Rate increase relative to 0.18M</u> <u>NaCl reference condition</u>
A. <u>Sodium Chloride</u>	
0.18M	1
0.72M	5.8
1M	7
1.2M	7.7 Britten, et al.
1.85M	8.6
3.2M	12.3
4.75M	21
B. <u>Cesium Chloride</u>	
1M	7.6
4M	12.7
7.5M	15.6

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C. Sodium Phosphate

0.12(0.18M Na)	1
0.48M (0.72M Na)	5.6 Britten, et al. and
1M (1.5M Na)	8.4 Wetmur and Davidson
1.23M (1.85M Na)	10.1
2.1 (3.2M Na)	12.1

D. Sodium Perchlorate

1M	11
2.2M	6.8 Wetmur and Davidson
4.0M	3.4
5.2M	1.5
6.4M	0.7

E. Lithium Chloride

0.4M	3.9 Orosz and Wetmur
1M	11.6

F. Potassium Chloride

0.7M	5.3
1M	5.8
2M	5.4 Orosz and Wetmur
3M	10.0
4M	11

G. Sodium Bromide

3M	9 Orosz and Wetmur
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H. Sodium Sulfate

3M	9 Orosz and Wetmur
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I. Ammonium Chloride

4M

30

While the data in Table 1 relates to the rate increases found with respect to DNA:DNA reactions, it will be appreciated that the reaction rates of RNA with DNA are reported as being less affected by changes in salt concentration. Other researchers have demonstrated that for salt concentrations above the standard reference conditions, the relative rate of RNA:DNA reaction is affected to about one-half the extent of those rates found for DNA:DNA reactions when the RNA used has comparatively little secondary structure. For RNA reactants with more secondary structure, the effect of elevated salt concentration has been found to be even less. In fact, no change in rate is observed for hybridization of excess RNA with DNA over comparative ranges of salt concentrations (see, e.g., Van Ness, J. and Hahn, W. E. (1982) Nucl. Acids. Res. 10, 8061). While little data is available for RNA:DNA hybridization where the DNA is the excess reactant, it is commonly assumed in the art that the effect of elevated salt concentration on such a reaction system is comparable to that of the excess RNA system.

An alternative approach to the acceleration of the rate of these nucleic acid association reactions is the previously developed two-phase phenol aqueous emulsion technique for the re-association of DNA to DNA (Kohne, D. E., Levinson, S. A., and Byers, M. J. (1977) Biochemistry 16, 5329). In this two-phase system, the agitation of an emulsion formed between phenol and an aqueous salt solution has produced greatly accelerated reaction rates over 100 times faster than comparative standard condition rates. However, the two-phase phenol emulsion technique has not produced similarly greatly accelerated reaction rates for RNA:RNA



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and RNA:DNA systems. The greatest reaction rate increase observed for RNA:RNA and RNA:DNA reactions is only 50 to 100 times that of the standard reference condition rate. This technique is further limited in that reaction will not occur unless an emulsion is present and agitated and the reaction temperature is below 75°C.

A number of other techniques for producing reaction rate increases on the order of 10 fold above the standard reference condition rate have utilized the volume exclusion principle to promote the acceleration of the reaction rate. These techniques utilize the synthetic polymers polyethylene glycol, dextran, or dextran sulfate to reduce the volume of reaction solution available to the nucleic acid reactants and thereby increase their effective concentration. However, while reaction rate increases of 10 to 15 fold over the standard reference condition rate for DNA:DNA reactions have been reported, rate increases of only about 3 fold are reported for RNA:DNA reactions. Details of these techniques can be found in the following publications:

Renz, M., and Kurz, C. (1984) Nucl. Acids Res. 12, 3435;  
20 and

Wahl, G. M., Stern, M., and Stark, G. R. (1979) Proc. Natl. Acad. Sci. USA 75, 3683.

Accordingly, it is a principal object of the present invention to provide a method for the renaturation, reassociation, or hybridization of nucleic acids that produces a greatly accelerated reaction rate on the order of 100 or more times that of the standard reference condition rate and that is applicable to DNA:DNA, RNA:DNA, or RNA:RNA reaction systems. Additionally, it is a further object of the present invention to provide a method that promotes greatly accelerated reaction rates without requiring the utilization of a two-phase system or the formation

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of an emulsion. It is a further object of the present invention to provide a method wherein greatly accelerated reaction rates are obtainable without the need to increase the concentrations of single stranded nucleic acid reactants. Lastly, it is an additional object of the present invention to provide a method for greatly accelerating the rate of these nucleic acid association reactions that is widely applicable to a variety of reaction mixture volumes and hybridization temperatures.

10

SUMMARY OF THE INVENTION

Generally stated, the present invention accomplishes the above described objectives by providing a method for the formation of double stranded nucleic acid molecules from separate single stranded nucleic acid molecules wherein a single phase reaction solution incorporating a known concentration of at least one nucleic acid precipitating agent is utilized to greatly increase the reaction rate over the standard reference condition reaction rate. The improved method of the present invention is widely applicable to a broad range of reaction solution volumes and nucleic acid concentrations and promotes reaction rates on the order of 100 to 1000 fold greater than the standard reference condition reaction rate for DNA:DNA, RNA:DNA and RNA:RNA reactions.

More particularly, the method of the present invention comprises the steps of preparing an aqueous reaction solution containing complementary single stranded nucleic acids, one of which preferably incorporates a detectable marker, and a known concentration of at least one nucleic acid precipitating agent. The aqueous reaction solution so prepared is incubated at a temperature at which hybridization can occur and then assayed for the presence of double stranded nucleic acid molecules.

Additionally, alternative methods of the present invention are disclosed wherein the aqueous reaction solution also contains a known concentration of a nucleic acid denaturing agent and also where the nucleic acid precipitating agent is contained  
5 in a second solution which is added to the aqueous reaction solution before the incubation step.

The nucleic acid precipitating agents which are utilized to practice the various alternative methods of the present invention are preferably selected from the group consisting of detergent, dihydroxybenzene, sodium dodecyl sulfate, sodium diisobutyl  
10 sulfosuccinate, sodium tetradecyl sulfate, Sarkosyl, and the alkali metal salts and ammonium salts of  $\text{SO}_4$ ,  $\text{PO}_4$ ,  $\text{Cl}$ , and  $\text{HCOO}$ . The salt concentrations preferably range from about 1M to about 10M. Additionally, it is preferred that the aqueous  
15 reaction solutions are prepared to have a pH ranging from about 4 to 11 and the concentration of the organic compound strong nucleic acid precipitating agents preferably ranges from approximately 5 volume % to 95 volume % and the preferred concentration of the nucleic acid denaturing agents ranges from  
20 approximately 5 volume % to 95 volume %.

Incubation temperatures preferably range from just below the  $T_m$  of the double stranded nucleic acid association product to temperatures approaching room temperature of approximately 22°C. It will be appreciated that the addition of nucleic acid denatur-  
25 ing agents to the aqueous reaction solution will lower the temperature at which hybridization occurs. The hybridization temperatures for most reactions utilizing the methods of the present invention will range from approximately room temperature to 90°C.

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After incubation, the reaction solution is assayed through a variety of known assay techniques to detect the presence of the double-stranded nucleic acid product. A preferred assay procedure utilizes hydroxyapatite (HA) for this purpose.

5 Further objects, features and advantages of the method of the present invention will become apparent to those skilled in the art from a consideration of the following detailed description. The following abbreviations are offered as an aid to understanding the specification.

10

ABBREVIATIONS

PB	Sodium phosphate solution. (A mixture of equimolar amounts of $\text{Na}_2\text{HPO}_4$ and $\text{Na H}_2\text{PO}_4$ .)
PK	Proteinase K
EGTA	Ethylene glycol bis- $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid
HA	Hydroxyapatite
15 STDS	Sodium tetradecyl sulfate
SDIBSS	Sodium diisobutyl sulfosuccinate
Tris	Tris(hydroxymethyl)aminomethane hydrochloride
G HCl	Guanidine hydrochloride
DNA	Deoxyribonucleic acid
20 RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
cDNA	Complementary DNA
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate

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DETAILED DESCRIPTION OF THE INVENTION

In a broad aspect, the method of the present invention is based upon the surprising discovery that relatively high concentrations of nucleic acid precipitating agents (both salts and organic compounds) will greatly accelerate the rate at which single stranded nucleic acid molecules with regions of complementary base sequences will combine to form base paired double stranded nucleic acid molecules. Reaction rates are increased as much as 800 times over the standard references condition rate for DNA:DNA reactions and as much as 3000 times over the standard reference condition rate for RNA:DNA reactions and as much as 1000 times over the standard reference condition rate for RNA:RNA reactions. What is more, these greatly increased reaction rates occur in a one-phase system and no emulsion or shaking is required.

Such significant increases in the rate of these reactions comes in complete contrast to the teaching of the prior art. For example, an often used accelerated reaction condition is approximately 1M NaCl (or an equivalent to it) which produces a reaction rate approximately 8 to 25 times faster than the reference standard condition. Such a rate increase is not the "greatly accelerated" rate disclosed by the present invention. As shown by the prior art table discussed above, increasing the concentration of NaCl to 4.75M results in little more of a rate increase. Similarly, concentrations of CsCl (also commonly used to promote increased reaction rates) up to 7.5M produce analogous increases of approximately 15 times that of the standard reference condition rate. Similar rate increases over the standard reference condition rate were also found for 1M  $(\text{NH}_4)_2\text{SO}_4$  and 1M LiCl, each salt producing rate increases of approximately 13 fold

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to 18 fold, which is roughly comparable to the rate increase observed for 1M NaCl or CsCl. However, in contrast to these known results, it was surprisingly discovered that increasing the concentration of  $(\text{NH}_4)_2\text{SO}_4$  to 2M greatly increased the reaction rate by an additional factor of 33 to approximately 600 times that of the standard reference condition rate. Additional research disclosed a comparable rate increase when the concentration of LiCl was increased to 4M wherein a rate increase of an additional 30 fold was observed, producing an accelerated reaction rate 467 times greater than that of the standard reference condition.

Comparable patterns of great acceleration were also discovered for ammonium formate, cesium sulfate, sodium sulfate, lithium sulfate, sodium phosphate and several detergents and organic compounds.

These same factors (aggregation or precipitation) which prevented the earlier researchers from discovering the method of the present invention are also proposed as being responsible for the greatly accelerated reaction rates of the methods of the present invention. It is hypothesized that nucleic acid precipitating agents cause the single stranded nucleic acid molecules to aggregate and thereby stimulate the reaction rate. As discussed above, the rate at which a given pair of complementary single stranded nucleic acids will form double stranded nucleic acid molecules is directly related to their concentration in the reaction solution. The higher the nucleic acid concentration, the faster the rate of reaction. In the presence of a nucleic acid precipitating agent, the single stranded nucleic acid molecules aggregate or associate together in solution. This aggregation or semi-precipitation results in high concentrations of nucleic acid

in localized regions of the reaction solution. If the aggregation occurs at a temperature where hybridization or reassociation can occur, the rate of the reaction is greatly increased.

In order for the complementary single stranded nucleic acid molecules to reassociate or hybridize together in a reaction solution containing a nucleic acid precipitating agent, the temperature must be high enough for the reaction to occur. Renaturation, reassociation or hybridization usually occurs at optimal rates at roughly 10°C to 30°C below the  $T_m$  of the double stranded nucleic acid molecule involved. In the reaction solution of the present invention, the  $T_m$  of most double stranded nucleic acid molecules will range from 85°C to 100°C. When the reaction solution of the present invention contains one or more nucleic acid denaturing agents, the  $T_m$  will be greatly lowered and temperatures as low as room temperature can be utilized to achieve optimum reaction rates. Accordingly, reaction temperatures of approximately 20°C to 90°C should produce optimum rates of reaction.

As discussed above, a variety of nucleic acid precipitating inorganic salts have been discovered to greatly increase the rate of reaction when used at sufficiently high concentrations. In general, the salts which have been effective for the method of the present invention are those which contain at least one of the stronger salting out cation or anion groups (namely  $SO_4$ ,  $PO_4$ , Li,  $NH_4$ ). Additionally, organic compounds which are miscible with the reaction solution and which have precipitating or salting out properties are also effective in promoting greatly accelerated reaction rates. Examples of such compounds include detergent, dihydroxybenzene, Sarkosyl, sodium dodecyl sulfate, sodium diisobutyl sulfosuccinate and sodium tetradecyl sulfate.

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To determine which salts or other compounds possess the requisite nucleic acid precipitating properties to practice the method of the present invention, it is necessary to first screen the compounds to determine if the compounds will precipitate single stranded nucleic acid molecules and then to determine whether the precipitate so formed will disappear when the reaction solution is heated to a temperature where reaction can occur. The nucleic acid precipitating agents are then analyzed to determine the preferred concentrations and incubation temperatures for producing optimal reaction rate increases. This screening procedure also makes it possible to determine the effective concentration of the nucleic acid precipitating agent necessary to promote greatly increased reaction rates.

For example, Sarkosyl (N-lauroylsarcosine sodium salt) was screened for its ability to increase nucleic acid reassociation rates in the following manner. First, a series of solutions containing known amounts of purified liver RNA (final concentration at 4 mg/ml) and varying amounts of Sarkosyl (ranging from approximately 9 volume % to 24 volume %) were prepared. The solutions were thoroughly mixed and checked for the presence of a precipitate using either direct visual observation or a spectrophotometer using a wavelength at which neither the Sarkosyl or the nucleic acid absorbs. If a precipitate was observed in a solution, the solution was heated to approximately 40°C to 90°C to determine whether the degree of precipitation would change. It was found that at 10% to 14% Sarkosyl, little or no precipitation of the nucleic acid was observed. However, at higher concentrations, Sarkosyl was found to precipitate nucleic acid. A number of further experiments were then conducted to determine the preferred concentration and incubation temperatures for producing optimal reaction rate increases.



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The following tables are an illustrative listing of the reaction rate increases that can be expected with a variety of concentrations of preferred inorganic salt nucleic acid precipitating agents.

5

TABLE 2

THE EFFECT OF HIGH CONCENTRATIONS OF  
CERTAIN SALTS ON DNA:DNA HYBRIDIZATION RATE

	<u>Salt</u>	<u>Rate increase relative to 0.18M Na reference condition</u>
	A. <u>Ammonium Sulfate</u>	
	1M	17.5
10	2M	600
	2.1M	600
	2.5M	467
	3.1M	70
	B. <u>Lithium Chloride</u>	
15	1M	13
	3.5M	66
	4M	467
	5M	280
	6M	19
20	C. <u>Other Salts</u>	
	2M Cesium Sulfate	280
	1.9M Sodium Sulfate	600
	2M Lithium Sulfate	420
	6M Ammonium Formate	210
25	2.4M Sodium Phosphate	800

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TABLE 3

EFFECT OF HIGH CONCENTRATIONS OF  
SALTS ON EXCESS RNA:DNA HYBRIDIZATION RATES

	<u>Salt</u>	<u>Rate increase relative to reference 0.18M Na condition</u>
	0.18M Sodium Chloride	1
5	0.72M Sodium Chloride	3.6
	2M Ammonium Sulfate	1500
	2.4M Sodium Phosphate	3000
	4M Lithium Chloride	600
	2M Sodium Sulfate	3460
10	Ammonium Sulfate	
	1M	90
	2M	1500
	3M	600

With this understanding of the nucleic acid precipita-  
 15 ting agents, the method of the present invention is as follows.  
 The first step of the preferred method is the preparation of an  
 aqueous reaction solution containing a quantity of a first single  
 stranded nucleic acid molecule and a quantity of a second single  
 stranded nucleic acid molecule, preferably incorporating a detec-  
 20 table marker and at least one segment of base sequences which are  
 complementary to a corresponding segment of base sequences of the  
 first single stranded nucleic acid molecule. Additionally, a  
 known concentration of at least one of the previously discussed  
 nucleic acid precipitating agents is also incorporated into the  
 25 aqueous reaction solution in a concentration sufficient to  
 greatly accelerate the rate of reaction by a factor of at least

50 to 100 times the rate of the standard reference condition reaction. Outside of practical consideration such as the solubility limit of the single stranded nucleic acid reactants, there is no real limit as to the volume of aqueous reaction solution that may be utilized to practice the method of the present invention or to the quantity of single strand nucleic acid molecule reactants as well. Additionally, while it should be emphasized that the nucleic acid precipitating agent is all that is necessary to obtain the greatly increased reaction rates, additional additives may be incorporated into the aqueous reaction solution such as buffers, EGTA, EDTA, SDS, SK, PK, ETOH, Urea, Guanidine HCL, Glycogen and dilute Amphyl. Additionally, it should be noted that while it is preferred that at least one of the single stranded nucleic acid molecule reactants incorporates a detectable marker, the marker is not essential to promoting the greatly accelerated reaction rates.

The next step of the method of the present invention is to incubate the aqueous reaction solution. As discussed above, temperatures ranging from just below the  $T_m$  to approximately room temperature are sufficient for incubating the reaction solution. The actual temperature utilized will vary depending on the concentrations of the reactants and whatever additional additives are incorporated into the reaction solution. However, most reactions will be conducted at incubation temperatures ranging from approximately room temperature to 90°C.

The last step in the method of the present invention is to assay the incubated aqueous reaction solution for the presence of double stranded nucleic acid molecules. A wide variety of assaying techniques are known in the art and are contemplated as being within the scope of the present invention. A preferred assaying technique involves the removal of an aliquot from the

incubated reaction solution at a specified time after the start of the reaction. The aliquot is diluted into 1 ml of 0.14M PB, 0.02% sodium dodecyl sulfate (SDS). The diluted solution is then passed over a column of hydroxyapatite (HA) (bed volume equaling 1 ml) which has been preequilibrated to 0.14M PB, 0.02% SDS at 67°C. Single stranded DNA molecules will not bind to the HA, but RNA and double stranded nucleic acid molecules will be adsorbed to the column. Nonhybridized single stranded nucleic acid molecules are then removed from the column by passing 5 ml of column buffer 0.14M PB, 0.02% SDS over the column. The adsorbed nucleic acid is recovered from the column by eluting the column with 0.3M PB at 67°C. The various solution fractions so produced may then be assayed for the detectable marker (such as radioactive hydrogen or iodine).

15 An alternative preferred method of assaying the incubated aqueous reaction solution for the presence of double strand nucleic acid follows.

(a) Remove an aliquot from the solution to be tested and mix with 5ml of 0.14M PB, 0.02% SDS containing one 0.1 gm of 20 HA. Vortex the mixture for 5-10 seconds.

(b) Incubate the mixture at 72°C for 5 minutes.

(c) Centrifuge the mixture in a table top centrifuge for 1 minute to pellet the HA. Discard the supernate fraction.

(d) Add 5ml of 0.14M PB, 0.02% SDS to the tube and 25 vortex to resuspend the HA.

(e) Repeat (c).

(f) Assay the HA for detectable marker.

An alternative approach for practicing the method of the present invention incorporates the additional step of mixing a 30 second solution containing the nucleic acid precipitating agent into the previously prepared aqueous reaction solution prior to

incubating the resultant mixture. Thus, the alternative method comprises the steps of preparing the previously discussed aqueous reaction solution, mixing the aqueous reaction solution with a second solution containing a known concentration of at least one  
5 nucleic acid precipitating agent which is miscible with the aqueous solution and capable of precipitating single stranded nucleic acid molecules from an aqueous solution, incubating the resulting mixture at the previously discussed temperatures, and assaying the incubated mixture for the presence of double  
10 stranded nucleic acid molecules. This alternative method serves to eliminate any problems which may occur with the premature aggregation of the single stranded nucleic acid molecule reactants in the aqueous reaction solution.

An additional modification to both of the alternative  
15 methods for practicing the accelerated rate reaction of the present invention involves the addition of a known concentration of at least one nucleic acid denaturing agent such as alcohol to the aqueous reaction solution. Preferably the concentration of denaturing agent added will range from approximately 5% by volume  
20 to approximately 95% by volume. For example, alcohol is a denaturant and functions to lower the temperature at which the reaction will occur. Ethanol is soluble in 2M  $(\text{NH}_4)_2\text{SO}_4$  to approximately 20%. At this concentration, the reaction will occur at a temperature of approximately 49°C instead of the usual  
25 60°C to 80°C.

Another denaturing agent which can be added to the aqueous reaction mixture is Urea. The presence of Urea in the reaction mix has little effect on the extent or rate of hybridization in several of the accelerated rate systems checked thus far.  
30 Example 36 is an example of one such system. Urea is an excellent solubilizing agent for many non-nucleic acid compounds which

may be present in a sample and is useful to minimize any effect these compounds might have on the hybridization reaction. Preferably, the concentration of Urea present in the reaction mixture will be approximately 0.01 to about 4M. The actual amount of Urea to be used must be determined for each different situation.

Guanidine HCl (GHCl) is another denaturing agent which can be added to the aqueous reaction mixture. This agent is useful to solubilize non-nucleic acid substances which may otherwise interfere with the hybridization reaction. Addition of GHCl to an aqueous reaction mixture optimized for both rate and extent of hybridization seen at specific times of incubation. A higher concentration of accelerating agent must be used in order to optimize the extent of hybridization when GHCl is present. Example 46 presents data concerning this. It is likely that the GHCl solubilizes nucleic acids to a certain extent and that more accelerating agent is needed to concentrate the nucleic acids for rapid hybridization. A similar situation occurs with the sodium phosphate system as seen in Example 46.

Regardless of which of the alternative methods is utilized to practice the method of the present invention, the nucleic acid precipitating agents are preferably selected from the group consisting of detergent, dihydroxybenzene, sodium dodecyl sulfate (SDS), sodium diisobutyl sulfosuccinate (SDIBSS), sodium tetradecyl sulfate (STDS), Sarkosyl, and the alkali metal salts and ammonium salts of  $\text{SO}_4$ ,  $\text{PO}_4$ ,  $\text{Cl}$ , and  $\text{HCOO}$ . It is also contemplated as being within the scope of the present invention to combine various members of this group in a single aqueous reaction solution. Additionally, it is also contemplated as being within the scope of the present invention to utilize a variety of detergent agents in addition to the organic compounds

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disclosed. Accordingly, those compounds specifically disclosed and claimed in the present invention are those which are currently known to be suitable for practicing the method of the present invention. Analogous compounds are therefore considered to be  
5 within the scope of the present invention.

It will be appreciated that the effective concentrations of nucleic acid precipitating agents necessary to practice the method of the present invention will vary with the amount of nucleic acid as well as with the size of the nucleic acid in the  
10 reaction solution and the pH of the solution as well as with the presence of other compounds. Accordingly, it is preferred that the concentrations will range from approximately 1M to 10M for the inorganic salt compounds and from approximately 5% by volume to approximately 95% by volume for the organic compounds.  
15 Additionally, it is preferred that the pH of the reaction solution will range from approximately 4 to 11.

Lastly, as discussed above, the preferred incubation temperature for the aqueous reaction solution should range from approximately room temperature to approximately 90°C.

20 The method of the present invention is suitable for bacterial, viral, mammalian and chemically synthesized nucleic acid. The completeness of the reaction will vary depending upon the concentration of the strong nucleic acid precipitating agent as well as on the amount of nucleic acid in the original reaction  
25 solution and on the composition of the reaction mixture. At low concentrations of nucleic acid, well over 90% of the single stranded nucleic acid will associate to form double stranded nucleic acid molecules. At higher nucleic acid concentrations, the completeness of the reaction will only be approximately 70%  
30 or less even though the rate of the reaction will be greatly increased. It should be noted that at very high concentrations

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of nucleic acid reactants, the rate of reaction will be accelerated to a lesser degree.

The amount of single stranded nucleic acid molecule reactants present in the aqueous reaction solution can range from an upward extreme approaching the solubility limit of the nucleic acid molecules to a lower extreme on the order of  $10^{-9}$  micrograms. Interestingly, the reaction rate increase for high concentrations of DNA:DNA or DNA:RNA reactions is lower than that for low concentrations of DNA:DNA and RNA:RNA reactions. Thus, the method of the present invention is applicable to both high and low concentrations of reactants. Along these lines, preferred reaction solution volumes will be on the order of a milliliter or less to a fraction of a microliter. However, it should be emphasized that other reaction solution volumes are contemplated as being within the scope of the present invention. Additionally, while the presence of small quantities of protein and other cell components will not greatly interfere with the reaction of the method of the present invention, excess heterologous RNA or DNA or other cellular components will slow the reaction rate to various extents as well as affecting the completeness of the hybridization.

Detergents are useful to help minimize the effect of excess cell and other components on the reaction of the method of the present invention. Addition of detergents compatible with individual rate accelerating agents to reaction mixtures is helpful in this regard. Certain detergents greatly accelerate nucleic acid hybridization and are quite useful in this regard. The optimum rate accelerator concentration to be used for different nucleic acids is dependent on the variables discussed above.



It should also be noted that the greatly accelerated reaction rates have been achieved for nucleic acid molecules ranging from approximately 30 bases long to molecules on the order of  $10^4$  bases long. However, the method of the present invention is contemplated as being applicable to nucleic acid molecules ranging from short molecules on the order of 10 to 15 bases long to longer molecules in excess of  $10^4$  bases long.

The following examples are offered as being illustrative of the method of the present invention and not by way of limitation.

The methods of the invention described in the above examples have great significance in the area of detection and identification of a wide variety of different life forms in various types of samples. The said methods make it possible to detect and identify many life forms with a rapidity and sensitivity heretofore unattainable. The following examples illustrate this.

#### EXAMPLE 1

##### Method for Using Sodium Phosphate to Increase DNA:DNA Hybridization Rates

##### 1. Mix thoroughly:

50 microliters of 0.17% SDS,  $3 \times 10^{-3}M$ , EDTA, containing 0.004 mcg of sonicated single strand  $^3H$  -E.Coli. DNA of about 300 to 700 bases in length.

25 +50 microliters of 4.8M sodium phosphate pH = 6.8

2. Incubate the mixture at  $76^\circ C$  and remove aliquots at specified times after the start. Dilute the aliquots into 0.14M

PB 0.02% SDS and assay for hybridization using hydroxyapatite as described earlier.

The above procedure results in a rate increase of about 800 relative to the rate at the standard reference condition.

5 Such large rate increases can be attained with a variety of different volumes, concentrations of sodium phosphate, DNA concentrations, EDTA and SDS concentrations and temperatures of incubation.

#### EXAMPLE 2

10 Method for Using Sodium Sulfate to Increase DNA:DNA

##### Rates

1. Mix thoroughly:

0.15 ml water containing 2 mcg of  $^3\text{H}$  - E.Coli. sonicated single strand DNA (300 to 700 bases long)

15 + 0.85 ml of 2.25M Sodium sulfate.

2. Incubate the mixture at 77°C and remove aliquots at specified times after the start. Dilute the aliquot into 1 ml 0.14M PB, 0.02% SDS and assay on HA as described earlier.

This procedure results in a rate increase of about 600  
20 fold relative to the reference condition.

#### EXAMPLE 3

LiCl Rate Increase Method for DNA:DNA Hybridization

1. Mix thoroughly:

0.3ml 9.16M Tris pH=7.8, containing about 10 mcg sonica-  
25 ted single strand  $^3\text{H}$  - E.Coli. DNA (about 300 to 700 bases long)  
+ 0.2 ml 10M lithium chloride.

2. Incubate the mixture at 76°C and remove aliquots at specified times after the start. Dilute the aliquot and assay for hybridization as described earlier.

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This procedure results in a rate increase of about 600 relative to the reference condition.

EXAMPLE 4

Ammonium Sulfate Method for DNA:DNA Hybridization Rate

5 Increase

A. Bacterial DNA

1. Mix thoroughly:

50 microliters of 0.2 Tris pH = 7.8, containing about 5 mcg of sonicated single strand  $^3\text{H}$  - E.Coli. DNA (about 10 300 to 700 bases long);

+ 50 microliters of 4.0M Ammonium sulfate.

2. Incubate the mixture at 76°C and remove aliquots at specified times after the start. Dilute the aliquot and assay for hybridization as described earlier.

15 This procedure resulted in a rate increase of about 700 relative to the reference condition.

EXAMPLE 5

B. Bacterial DNA: Ethanol Modification

The temperature at which the hybridization is conducted 20 can be lowered dramatically by adding alcohol to the reaction mixture. Ethanol is soluble to about 20% in 2M Ammonium sulfate.

1. Mix thoroughly:

0.05 ml of 34% ethanol in water containing 0.4 mcg of sonicated single strand  $^3\text{H}$  - E.Coli. DNA (300 to 700 bases 25 long).

+0.05 ml 4M ammonium sulfate, 0.01M EDTA, 0.01M PH

pH = 6.8

2. Incubate the mixture at 49°C for appropriate times and remove aliquots. Dilute and assay the aliquots for hybridization as described earlier.

This procedure results in a rate increase of about 100 fold relative to the reference condition.

#### EXAMPLE 6

##### C. Mammalian DNA: Low Concentration

1. Mix thoroughly: 0.1 ml of 0.02M EDTA containing 26 mcg of sonicated single strand <sup>3</sup>H DNA (about 10 400 to 800 bases long):

+0.1 ml 4M Ammonium sulfate, 0.1M PB pH = 6.8

2. Incubate the mixture at 68°C and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

15 This procedure results in a rate increase of about 200 fold relative to the reference condition.

#### EXAMPLE 7

##### D. Mammalian DNA: Low concentration: Ethanol Modification

20 1. Mix thoroughly:  
0.05 ml of 0.016M EDTA, 40% ethanol in water containing 26 mcg sonicated single strand <sup>3</sup>H human DNA (about 400 to 800 bases long);

+0.05 ml 4M ammonium sulfate, 0.1M PB pH = 6.8

25 2. Incubate the mixture at 49°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure resulted in a rate increase of about 180 fold relative to the reference condition.

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EXAMPLE 8

E. Mammalian DNA: High Concentration

1. Mix thoroughly:

0.025 ml of 0.04M EDTA in water containing 130 mcg  
5 of sonicated single strand human  $^3\text{H}$  DNA (about 400 to 800 bases  
long);

+0.025 ml 4M ammonium sulfate, 0.1M PB pH = 6.8

2. Incubate the mixture at 68°C and at specified  
times remove aliquots. Dilute each aliquot and assay for  
10 hybridization as described earlier.

This procedure results in a rate increase of about 90  
fold relative to the reference condition.

EXAMPLE 9

F. Mammalian DNA: High Concentration: Ethanol

15 Modification

1. Mix thoroughly:

0.0125 ml of 0.016M EDTA in water containing 40%  
ETOH and 65 mcg of sonicated single strand human  $^3\text{H}$  DNA (400 to  
800 bases long);

20 + 0.0125 ml 4M ammonium sulfate, 0.1M PB pH = 6.8

2. Incubate the mixture at 49°C and at specified times  
remove aliquots. Dilute each aliquot and assay it for  
hybridization as described earlier.

This procedure results in a rate increase of about 130  
25 fold relative to the reference condition.

EXAMPLE 10Purified RNA:DNA Hybridization Rate Increases with  
Sodium PhosphateA. Excess RNA: 0.2 ml Volume

5

## 1. Thoroughly mix:

0.1 ml of 0.2% SDS,  $10^{-3}$ M EDTA in water containing  
 $2 \times 10^{-3}$  mcg of Polio I RNA and  $2 \times 10^{-4}$  mcg of  $^3\text{H}$ -cdNA, 300 to 600  
complementary to Polio RNA, 300 to 600 bases long.

+ 0.1 ml 4.8M sodium phosphate pH = 6.8

10

2. Incubate the mixture at 76°C and at specified  
times remove aliquots. Dilute each aliquot and assay it for  
hybridization as described earlier.

This procedure results in a hybridization rate increase  
of 3300 fold over the rate in the standard reference condition of  
15 60°C, 0.18M Na.

EXAMPLE 11B. Excess RNA: 1 ml volume

## 1. Thoroughly mix:

0.5 ml  $10^{-3}$  EDTA, 0.2% SDS containing  $2 \times 10^{-3}$  mcg  
20 Polio I RNA and  $2 \times 10^{-4}$  mcg of 300 to 600 base long Polio  $^3\text{H}$ -cdNA;  
+ 0.5 ml 4.8M sodium phosphate pH = 6.8

2. Incubate at 76°C and at specified times remove  
aliquots. Dilute each aliquot and assay it for hybridization as  
described earlier.

25

This procedure results in a hybridization rate increase  
of 3300 fold over the reference condition rate.

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EXAMPLE 12

C. Excess RNA: Plus Added Heterologous High Molecular Weight RNA

1. Thoroughly mix:

5 0.05 ml  $10^{-3}$ M EDTA, 0.4% SDS in water containing  $2 \times 10^{-3}$  mcg Polio I RNA,  $2 \times 10^{-4}$  mcg Polio I  $^3\text{H}$ -cDNA (300 to 600 bases long) and 5 mcg. of calf liver RNA;

+ 0.05 ml 5.1 sodium phosphate.

2. Incubate the mixture at  $76^\circ\text{C}$  and at specified times  
10 remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure resulted in a rate increase of 600 fold over the rate at the reference condition.

EXAMPLE 13

15 D. Excess DNA:

1. Thoroughly mix:

0.012 ml 0.012% Sarkosyl containing  $1.5 \times 10^{-6}$  mcg of  $^3\text{H}$ -cDNA and  $1.2 \times 10^{-6}$  ribosomal RNA from Legionella pneumophila. The  $^3\text{H}$  cDNA (100 to 300 bases long) is complementary to  
20 only about one third of the RNA. The cDNA/RNA ratio is about 4/1 for the complementary RNA and DNA sequences.

+ 0.02 ml of 4.8M sodium phosphate pH = 6.8

2. Incubate the mixture at  $76^\circ\text{C}$  and at specified times remove aliquots. Dilute each aliquot and assay it for  
25 hybridization.

This procedure results in a rate increase of greater than 3000 over the rate at the reference condition.

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EXAMPLE 14

E. Excess DNA: Non Purified RNA

1. Thoroughly mix:

0.012 ml of  $1.4 \times 10^{-3}M$  EDTA,  $1.4 \times 10^{-3}$  EGTA, 0.012

5 SDS, 0.3% Sarkosyl containing 10 mcg Proteinase K, 1.0 ml

$^3H$ -cDNA (100 to 300 bases long) complementary to Legionella  
pneumophila ribosomal RNA and 4400 Legionella pneumophila  
bacteria which contain about  $6 \times 10^{-5}$  mcg. of ribosomal RNA.

+0.02 ml of 4.8M sodium phosphate pH = 6.8

10 2. Incubate the mixture at 76°C and at specified times  
remove aliquots. Dilute each aliquot and assay each aliquot for  
hybridization as described earlier.

This procedure results in a rate increase of greater  
than 150 fold over the rate in the reference condition.

15

EXAMPLE 15

F. Excess RNA: Non Purified RNA

1. Thoroughly mix:

0.012 ml of 0.16% Sarkosyl containing  $10^{-5}$  mcg of

$^3H$ -cDNA (100 to 300 bases long) complementary to E. Coli  
20 ribosomal RNA and 5000 E. Coli bacteria which contain about  
 $7 \times 10^{-5}$  mcg ribosomal RNA. .

+0.02 ml of 4.8M sodium phosphate pH = 6.8

25 2. Incubate the mixture at 76°C and at specified times  
remove aliquots. Dilute each aliquot and assay it for  
hybridization as described earlier.

This procedure results in a rate increase of greater  
than 100 fold over the rate at the reference condition.



EXAMPLE 16

RNA:DNA Hybridization Rate Increase Promoted by Sodium

Sulfate

1. Mix well:  
5 0.023 ml of  $10^{-3}$ M EDTA, 0.1% SDS containing  $2 \times 10^{-3}$  mcg Polio I RNA and  $2 \times 10^{-4}$  mcg  $^3\text{H}$ -cDNA (300 to 600 bases long) which is complementary to Polio I RNA.  
+ 0.178 ml. 2.25M sodium sulfate.
2. Incubate at  $76^\circ\text{C}$  and at specified times remove  
10 aliquots. Dilute aliquot and assay it for hybridization as described earlier.

This procedure resulted in a rate increase of over 3000 relative to the reference rate.

EXAMPLE 17

RNA:DNA Hybridization Rate Increase Promoted by Ammonium

Sulfate

1. Mix well:  
0.1 ml of 0.2% SDS,  $10^{-3}$ M EDTA containing  $2 \times 10^{-3}$  mcg of  
20 Polio I RNA and  $2 \times 10^{-4}$  mcg of  $^3\text{H}$ -cDNA (300 to 600 bases long) which is complementary to Polio I RNA  
+ 0.1 ml of 4M ammonium sulfate.
2. Incubate at  $77^\circ\text{C}$  and at specified times remove  
aliquots. Dilute each aliquot and assay it for hybridization as  
25 described earlier.

This procedure resulted in a rate increase of about 1500 relative to the reference condition rate.

EXAMPLE 18RNA:RNA Hybridization Rate Increase Promoted by AmmoniumSulfate

## 1. Mix well:

0.1 ml 0.2% SDS,  $10^{-3}$ M EDTA containing  $4 \times 10^{-3}$  mcg VSV RNA and  $2 \times 10^{-4}$  mcg  $^{125}\text{I}$ -cRNA (about 300 to 800 bases long) which is complementary to VSV RNA.

+0.1 ml 4.4M ammonium sulfate.

2. Incubate at  $87^{\circ}\text{C}$  and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization by a standard ribonuclease technique.

This procedure resulted in a rate increase of over 1000 relative to the 0.18M Na rate.

EXAMPLE 19RNA:RNA Hybridization Rate Increase Promoted by Sodium15 Phosphate

## 1. Mix well:

0.1 ml 0.2% SDS,  $10^{-3}$ M EDTA containing  $4 \times 10^{-3}$  mcg VSV RNA and  $2 \times 10^{-4}$  mcg VSV  $^{125}\text{I}$ -cRNA which is complementary to VSV RNA.

20 +0.1 ml 4.8M sodium phosphate.

2. Incubate at  $83^{\circ}\text{C}$  and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization by a standard ribonuclease technique.

This procedure results in a rate increase of greater than 500 relative to the reference condition rate.

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EXAMPLE 20Method For Using Sodium Dodecyl Sulfate To Increase  
DNA:RNA Hybridization Rates

This example shows that a greatly accelerated  
5 hybridization rate occurs at 28.5% ( $\frac{W}{V}$ ) SDS when probe is in  
excess over purified RNA.

A. Excess Probe Plus Homologous RNA

1. Thoroughly mix:

1  $\mu$ l solution containing  $0.6 \times 10^{-4}$  micrograms  
10 Legionella Ribosomal RNA (rRNA).

1  $\mu$ l 5M Sodium Phosphate Buffer (pH=6.8)(PB).

3  $\mu$ l probe solution containing  $10^{-4}$  micrograms of  
r125 - cDNA complementary to about 1/5 of the Legionella rRNA  
sequence.

15 95  $\mu$ l 30% ( $\frac{W}{V}$ ) sodium dodecyl sulfate in  $H_2O$ .

2. Incubate the mixture at 72°C and at specific  
times remove aliquots. Dilute each aliquot and assay for  
hybridization as described earlier.

This procedure resulted in a rate increase of 100-200  
20 fold over the rate at the reference condition.

EXAMPLE 21B. Excess Probe Hybridization

This example shows that a greatly accelerated  
hybridization rate occurs at 31.4% ( $\frac{W}{V}$ ) SDS when probe is in  
25 excess over purified RNA.

1. Thoroughly mix:

1  $\mu$ l of solution containing  $1.6 \times 10^{-4}$  micrograms of  
Legionella Ribosomal RNA.

6  $\mu$ l  $H_2O$ .

30 1  $\mu$ l 5M Sodium Phosphate Buffer (pH=6.8)(PB)

2  $\mu$ l probe solution containing  $10^{-4}$  micrograms of  $\text{I}^{125}$  - cDNA complementary to about 1/5 of the Legionella Ribosomal RNA sequence.

90  $\mu$ l 34.9% ( $\frac{W}{V}$ ) sodium dodecyl sulfate in  $\text{H}_2\text{O}$ .

- 5           2. Incubate the mixture at  $72^\circ\text{C}$  and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

This procedure resulted in a rate increase of 150-200 fold over the rate at the reference condition.

10

EXAMPLE 22

C. Excess RNA Hybridization:

This example shows that a greatly accelerated hybridization rate occurs at 31.4% ( $\frac{W}{V}$ ) SDS when purified RNA is in excess.

15

1. Thoroughly mix:

2  $\mu$ l solution containing  $3.2 \times 10^{-4}$  micrograms

Legionella Ribosomal RNA.

1  $\mu$ l  $\text{H}_2\text{O}$ .

1  $\mu$ l 5.0M Sodium Phosphate Buffer (pH=6.8)(PB).

20

2  $\mu$ l probe solution containing  $2.5 \times 10^{-5}$  micrograms of  $\text{I}^{125}$  - cDNA complementary to the Legionella Ribosomal RNA.

95  $\mu$ l 34.9% ( $\frac{W}{V}$ ) sodium dodecyl sulfate in  $\text{H}_2\text{O}$ .

2. Incubate the mixture at  $72^\circ\text{C}$  and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

This procedure resulted in a rate increase of 150-200 fold over the rate at the reference condition.

EXAMPLE 23

D. Detection of Legionella Bacteria Present in a Liquid Sample.

This example shows that a greatly accelerated  
5 hybridization rate occurs at 31.4% ( $\frac{W}{V}$ ) SDS when bacteria are isolated out of a liquid sample and lysed. Accelerated hybridization occurs even in the presence of non-nucleic acid bacterial cell components.

1. (a) Centrifuge a sample known to contain  
10 Legionella organisms at 14,000 xg for 10 minutes, and then remove the supernatant.

(b) Resuspend the pellet in a lysing buffer and lyse the bacteria, thus freeing the nucleic acid.

(c) Thoroughly mix:

15 1  $\mu$ l of lysed bacteria solution in 5% sodium dodecyl sulfate, 0.05M Tris buffer pH=8.2.

1  $\mu$ l H<sub>2</sub>O.

1  $\mu$ l 5.0M Sodium Phosphate Buffer (PB)

20 2  $\mu$ l probe solution containing  $5 \times 10^{-5}$  micrograms of I<sup>125</sup> - cDNA complementary to the Legionella Ribosomal RNA.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

25 This procedure resulted in a rate increase of 100-200 fold over the rate at the reference condition.

EXAMPLE 24

E. Detection of Legionella Bacteria in a Sputum Sample Known To Contain Such Bacteria.

This example shows that a greatly accelerated  
 5 hybridization rate occurs at 23.4% ( $\frac{W}{V}$ ) SDS when the bacteria have been isolated out of a clinical sample, lysed and hybridized with no purification of RNA.

1. (a) Solubilize 1 ml sputum by adding 0.1ml of 0.1M DTT and centrifuge for 10' at 14,000 xg to pellet bac-  
 10 teria. Discard the supernatant.

(b) Resuspend pellet in lysing buffer and lyse Legionella bacteria to free nucleic acids.

(c) Thoroughly mix:  
 30  $\mu$ l of lysing solution (containing about  
 15 8000 lysed Legionella bacteria) composed of 11% sodium dodecyl  
 sulfate ( $\frac{W}{V}$ );  $3 \times 10^{-3}$ M EDTA; 0.003M Tris unbuffered.

1  $\mu$ l 5M Sodium Phosphate Buffer (pH=6.8)(PB).

2  $\mu$ l probe solution containing  $10^{-4}$  micrograms  
 of  $^{125}$ S - cDNA complementary to the Legionella rRNA.

20 67  $\mu$ l 34.9% ( $\frac{W}{V}$ ) SDS.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

This procedure resulted in a rate increase of 100-200  
 25 fold over the rate at the reference condition.

EXAMPLE 25

F. Detection of Legionella Bacteria in a Sputum Sample Known to Contain Such Bacteria

This example shows that a greatly accelerated  
 30 hybridization rate occurs at 23.4% ( $\frac{W}{V}$ ) SDS when the clinical

sample is assayed directly and the hybridization is done in the presence of the sputum components.

1. (a) Mix a sputum sample known to contain Legionella bacteria with an equal volume of a lysing agent solution (33% SDS, 0.01M unbuffered Tris, 0.01M EDTA, 0.01M EGTA). Incubate at 72°C 15 minutes.
- (b) Mix thoroughly:
  - 30  $\mu$ l of solution from 1(a).
  - 1  $\mu$ l 5M Sodium Phosphate Buffer (pH=6.8)(PB).
  - 2  $\mu$ l probe solution containing  $10^{-4}$  micrograms of  $I^{125}$  - cDNA complementary to the Legionella rRNA.
  - 67  $\mu$ l 34.9% ( $\frac{W}{V}$ ) SDS in  $H_2O$ .
2. Incubate the mixture at 72°C and at specified 15 times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

This procedure resulted in a rate increase of 100-200 fold over the rate at the reference condition.

#### EXAMPLE 26

#### 20 Method For Using Sodium Tetradecyl Sulfate (STDS) To Increase Nucleic Acid Hybridization Rate

This example shows a greatly accelerated rate occurs at 24.3% ( $\frac{W}{V}$ ) STDS when purified RNA is used.

#### A. RNA:DNA Hybridization in STDS.

- 25 1. Thoroughly mix:
  - 1  $\mu$ l of a solution containing  $1.7 \times 10^{-4}$  micrograms of Legionella rRNA.
  - 7  $\mu$ l  $H_2O$ .
  - 2  $\mu$ l probe solution containing  $10^{-4}$  micrograms
  - 30  $I^{125}$  - cDNA complementary to Legionella rRNA.

90  $\mu$ l 27% ( $\frac{W}{W}$ ) STDS, 0.03M PB; final pH=7.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

5 This procedure resulted in a rate increase of at least 500-1000 fold over the rate at the reference condition.

EXAMPLE 27

B. RNA:DNA Hybridization in STDS + UREA. Addition of Urea Increases The Extent of Hybridization.

10 This example shows that a greatly accelerated rate occurs at 24.3% ( $\frac{W}{W}$ ) STDS when Urea is present in the reaction mix.

1. Mix thoroughly:

1  $\mu$ l solution containing  $1.7 \times 10^{-4}$  micrograms of  
15 Legionella rRNA.

2  $\mu$ l H<sub>2</sub>O.

5  $\mu$ l 10M Urea.

2  $\mu$ l probe solution containing  $10^{-4}$  micrograms of  
I<sup>125</sup> - cDNA complementary to the Legionella rRNA.

20 90  $\mu$ l 27% ( $\frac{W}{W}$ ) STDS, 0.03M PB: final pH=7.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay for hybridization as described earlier.

This procedure resulted in a rate increase of at least  
25 500-1000 fold over the rate at the reference condition.

In addition a greater extent of hybridization is seen with urea present.



EXAMPLE 28

A. Hybridization Rate Increase Prompted by Sodium Diisobutyl Sulfosuccinate (SDIBSS).

This example shows that a greatly accelerated rate  
5 occurs at 41.4% ( $\frac{W}{V}$ ) SDIBSS when phosphate buffer (PB) is used to  
adjust the SDIBSS pH to about 7.

a. RNA:DNA hybridization

1. Mix well:

1  $\mu$ l of a solution containing  $1.7 \times 10^{-4}$   
10 micrograms of Legionella rRNA.

2  $\mu$ l 5M PB.

2  $\mu$ l  $I^{125}$  - cDNA complementary Legionella rRNA

4  $\mu$ l  $H_2O$ .

92  $\mu$ l 45% ( $\frac{W}{V}$ ) SDIBSS.

15 2. Incubate at 72°C and at specified times  
remove aliquots. Dilute each aliquot and assay for hybridization  
as described earlier.

This procedure resulted in a rate increase of 100-200  
fold over the rate at the reference condition.

EXAMPLE 29

B. RNA:DNA Hybridization in SDIBSS

This example shows that a greatly accelerated rate occurs at 33.8% (W SDIBSS when phosphate buffer is used to adjust the SDIBSS pH to about 7.

1. Mix well:

1  $\mu$ l solution containing  $1.7 \times 10^{-4}$  micrograms of Legionella rRNA.

2  $\mu$ l 5M PB.

10 2  $\mu$ l I<sup>125</sup> - cDNA complementary to Legionella rRNA.

20  $\mu$ l H<sub>2</sub>O

75  $\mu$ l SDIBSS 45% (W -).

15 2. Incubate at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure results in a hybridization rate increase of 100-200 fold over the reference condition rate.

EXAMPLE 30

C. RNA:DNA Hybridization in SDIBSS

20 This example shows that a greatly accelerated rate occurs at 38.2% (W SDIBSS when phosphate buffer is used to adjust the SDIBSS pH to about 7.

1. Mix well:

25 1  $\mu$ l of solution containing  $1.7 \times 10^{-4}$  micrograms Legionella rRNA.

2  $\mu$ l 5M PB

2  $\mu$ l I<sup>125</sup> - cDNA complementary to Legionella rRNA.

10  $\mu$ l H<sub>2</sub>O.

85  $\mu$ l 45% (W -) SDIBSS.

2. Incubate at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure results in a hybridization rate increase of 100-200 fold over the reference condition rate.

#### EXAMPLE 31

##### D. RNA:DNA Hybridization in SDIBSS.

A greatly accelerated rate occurs when unbuffered Tris is used to adjust the SDIBSS pH to about 9.

10 1. Mix well:  
1 µl of solution containing  $1.7 \times 10^{-4}$  micrograms of Legionella rRNA.

7 µl 1M Tris, unbuffered.

2 µl  $I^{125}$  - cDNA complementary to Legionella rRNA.

15 90 µl 45% ( - ) SDIBSS.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure resulted in a rate increase of 100-200 fold over the rate at the reference condition.

#### EXAMPLE 32

##### E. RNA:DNA Hybridization In A Mixture of Sodium Dodecyl Sulfate (SDS) and SDIBSS

A greatly accelerated rate occurs in SDS and SDIBSS.

1. Mix well:

1 µl of solution containing  $1.7 \times 10^{-4}$  micrograms Legionella rRNA.

7 µl 1M Tris unbuffered.

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2  $\mu$ l  $^{125}$ I - cDNA complementary to Legionella rRNA.

45  $\mu$ l 34.9% (  $\frac{W}{V}$  ) SDS.

45  $\mu$ l 45% (  $\frac{W}{V}$  ) SDIBSS.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure resulted in a rate increase of 100-200 fold over the rate at the reference condition.

#### EXAMPLE 33

#### 10 F. RNA:DNA Hybridization In SDIBSS In The Presence of Heterologous RNA

A greatly accelerated rate occurs in the presence of a high concentration (56 micrograms/ml) of heterologous RNA.

1. Mix well:

15 1  $\mu$ l of solution containing  $1.7 \times 10^{-4}$  micrograms Legionella rRNA.

3  $\mu$ l of solution containing 5.6 micrograms of heterologous bacterial RNA.

4  $\mu$ l 1M Tris, unbuffered.

20 2  $\mu$ l  $^{125}$ I - cDNA complementary to Legionella rRNA.

90  $\mu$ l 45% (  $\frac{W}{V}$  ) SDIBSS.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

25 This procedure resulted in a rate increase of at least 100-200 fold over the rat at the reference condition.

EXAMPLE 34

G. Detection of Legionella Bacteria In Sputum By  
RNA:DNA Hybridization In SDIBSS.

A greatly accelerated rate occurs when bacteria are  
5 isolated from sputum, lysed and hybridized in SDIBSS.

Hybridization occurred in SDIBSS even in the presence of non-  
nucleic acid cellular components.

1. (a) Solubilize 1 ml of sputum by adding 0.1ml  
0.25, DTT and centrifuge the mixture at 14,000 x g for 10' to  
10 pellet bacteria. Discard supernatant.

(b) Resuspend pellet in one third of its  
volume of lysing buffer (33% SDS, 0.01M EDTA, 0.01M Tris,  
unbuffered) and incubate at 72°C for 15' to lyse bacteria.

(c) Mix well:

15

2 µl 5M PB.

4 µl I<sup>125</sup> - cDNA complementary to Legionella  
rRNA.

30 µl 45% ( <sup>W</sup> - ) DBISS.  
<sup>W</sup>

20

(d) Add mixture (c) to mixture (b) and thor-  
oughly mix.

2. Incubate the mixture at 72°C and at specified  
times remove aliquots. Dilute each aliquot and assay it for  
hybridization as described earlier.

25

This procedure resulted in a rate increase of 5 fold  
fold over the rate of reference condition.

EXAMPLE 35

H. Detection of Legionella Bacteria In A Liquid Sample  
By RNA:DNA Hybridization In SDIBSS.

A greatly accelerated rate occurs when the bacteria are  
5 isolated from a non-clinical sample and hybridized in SDIBSS. A  
separate lysing incubation step was not performed.

1. (a) Prefilter the liquid sample known to  
contain Legionella to remove large particles.

(b) Mix well:

10 30  $\mu$ l solution of solution containing  
Legionella bacteria.

15 15  $\mu$ l 33% SDS, 0.01M Tris, unbuffered, 0.01M  
EDTA.

1.8  $\mu$ l 5M PB.

15 5  $\mu$ l I<sup>125</sup> - cDNA complementary to Legionella  
rRNA.

190  $\mu$ l 45% ( <sup>W</sup>- ) SDIBSS.

2. Incubate at 72°C and at specified times remove  
aliquots. Dilute each aliquot and assay it for hybridization as  
20 described earlier.

This procedure results in a hybridization rate increase  
of 100-200 fold over the reference condition rate.

EXAMPLE 36

I. RNA:DNA Hybridization In The Presence of SDIBSS and  
25 Urea.

A greatly accelerated rate occurs in a mixture of SDIBSS  
and Urea.

1. Mix well:

30 1  $\mu$ l of solution containing  $1.7 \times 10^{-4}$  micrograms of  
Legionella rRNA.

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2  $\mu$ l  $H_2O$ .

5  $\mu$ l 10M Urea.

90  $\mu$ l SDIBSS 45% (  $\frac{W}{W}$  ).

2. Incubate at 72°C and at specified times remove 5 aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure results in a hybridization rate increase of at least 200 fold over the reference condition rate.

#### EXAMPLE 37

#### 10 J. RNA:DNA Hybridization In SDIBSS and Urea

A greatly accelerated rate occurs in a mixture of SDIBSS and Urea.

1. Mix well:

2  $\mu$ l solution containing  $3.4 \times 10^{-4}$  micrograms of

15 Legionella rRNA.

1  $\mu$ l 5M PB.

2  $\mu$ l  $I^{125}$  - cDNA complementary to Legionella rRNA.

25  $\mu$ l 27% (  $\frac{W}{W}$  ) SDIBSS, 8M Urea.

70  $\mu$ l 45% (  $\frac{W}{W}$  ) SDIBSS.

20 2. Incubate at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This procedure results in a hybridization rate increase of 100-200 fold over the reference condition rate.

EXAMPLE 38K. RNA:DNA Hybridization In The Presence Of SDIBSS and NaSCN

A greatly accelerated rate occurs in a mixture of SDIBSS  
5 and sodium thiocyanate.

1. Mix well:

2  $\mu$ l solution containing  $3.4 \times 10^{-4}$  micrograms of  
Legionella rRNA.

1  $\mu$ l 5M PB.

10 2  $\mu$ l I<sup>125</sup> - cDNA complementary to Legionella rRNA.

5  $\mu$ l 10.5M NaSCN.

90  $\mu$ l 45% (  $\frac{W}{W}$  ) SDIBSS.

2. Incubate at 72°C and at specified times remove  
aliquots. Dilute each aliquot and assay it for hybridization as  
15 described earlier.

This procedure results in a hybridization rate increase  
of 100-200 fold over the reference condition rate.

EXAMPLE 39L. RNA:DNA Hybridization In Mixtures Of SDIBSS, Urea  
20 and NaSCN.

A greatly accelerated rate occurs in the presence of  
SDIBSS, sodium thiocyanate and Urea.

1. Mix well:

25 2  $\mu$ l of solution containing  $3.4 \times 10^{-4}$  micrograms of  
Legionella rRNA.

1  $\mu$ l 5M PB.

2  $\mu$ l I<sup>125</sup> - cDNA complementary to Legionella rRNA.

5  $\mu$ l 4M Urea, 4M NaSCN, 0.05 M DTT, 0.03M Hcl.

90  $\mu$ l 45% (  $\frac{W}{W}$  ) SDIBSS.



2. Incubate at 72°C and at specified times remove aliquots. Dilute aliquot and assay it for hybridization as described earlier.

This procedure results in a hybridization rate increase of at least 100-200 fold over the reference condition rate.

The following examples concern the rapid and sensitive detection of three medically important bacteria, each of which causes disease in humans.

A. Bacteria in the Mycobacteria group cause a variety of human disease. Prominent among them are tuberculosis and leprosy. Current diagnostic methods use the culture approach for the detection and identification of these bacteria. A growth step is necessary to amplify the bacteria so they can be detected and differential growth methods are used to identify these bacteria. This method of diagnosis is labor intensive and very slow. As it is important to know as soon as possible if the patient is infected with Mycobacteria in order to start the proper anti-microbial therapy, this method of diagnosis is labor intensive and very slow.

With current methods it takes 1-8 weeks to obtain a definitive diagnosis for Mycobacteria. The assay described in Example 44 takes about 2-3 hours to perform and does not require a growth step. Thus the method of the invention allows the design of a test for Mycobacteria which is over 100 times faster than the current methods, does not require a growth step, is much less labor intensive and is less expensive. In addition, the rapidity of the test will make rapid treatment of the disease possible with incalculable benefits to the patients.

B. The bacteria Mycoplasma pneumoniae also causes disease in humans. Culture methods are currently used for

diagnosis and a definitive diagnosis generally takes from 1-2 weeks.

The assay described in Example 42 takes less than 2 hours to perform and does not require a growth step. Thus the method of the invention allows the design of a test for Mycoplasma pneumoniae which is about 100 times faster than the current methods and has other advantages and benefits similar to those described in A above.

C. Legionella bacteria also cause human disease. Current recommended diagnostic procedures involve culture methods. Such methods generally yield a definitive answer in about 3 days but can take a week or longer.

The assay described in Example 44 takes less than 2 hours to perform, does not require a growth step, is about 20 times faster than current methods and has other advantages and benefits similar to those described in A above.

#### EXAMPLE 40

##### M. Detection Of Legionella Bacteria In Sputum Sample By RNA:DNA Hybridization In SDIBSS, Urea and SDS.

A greatly accelerated rate occurs when sputum is assayed directed in a mixture of SDIBSS, SDS and Urea.

1. (a) Mix well:

3  $\mu$ l sputum.

6  $\mu$ l 17% SDS, 0.01M Tris (unbuffered), 0.01M

25EDTA. Incubate at 72°C for 15'.

(b) Mix well:

3  $\mu$ l 1(a) solution.

2  $\mu$ l H<sub>2</sub>O.

3  $\mu$ l 10M Urea.

2  $\mu$ l  $I^{125}$  - cDNA complementary to Legionella

rRNA.

90  $\mu$ l 45% ( <sup>W</sup>- ) SDIBSS.

2. Incubate at 72°C and at specified times remove  
5 aliquots. Dilute each aliquot and assay it for hybridization as  
described earlier.

This procedure results in a hybridization rate increase  
of 100-200 fold over the reference condition rate.

#### EXAMPLE 41

- 10 N. Detection of Legionella Bacteria In Sputum Sample  
By RNA:DNA Hybridization In SDIBSS, Urea, SDS Mixes.

An example of the rapid detection of Legionella bacteria  
in a sputum sample by using a mixture of SDIBSS, SDS, and Urea to  
greatly accelerate hybridization rate.

- 15 1. (a) Mix well:

20  $\mu$ l of sputum.

80  $\mu$ l 5M Urea, 4M NaSCN, 0.05M Tris (unbuf-  
fered) and centrifuge for 10 minutes at 14,000 x g. Discard  
supernate.

- 20 (b) Add 25  $\mu$ l 11% SDS, 3.3M Urea, 0.007M  
EDTA, 0.05M Tris (unbuffered) to pellet and resuspend. Incubate  
at 72°C for 15 minutes.

- (c) Mix well:

5  $\mu$ l solution for 1(b).

- 25 2  $\mu$ l  $I^{125}$  - cDNA complementary to Legionella

rRNA.

3  $\mu$ l 10M Urea

90  $\mu$ l 45% ( <sup>W</sup>- ) SDIBSS.

2. Incubate the mixture at 72°C for one hour and  
30 assay for hybridization as described.

This procedure resulted in a rate increase of 100-200 over the rate at the reference condition.

EXAMPLE 42

O. Detection of Mycoplasma Pneumonia Bacteria In A  
5 Throat Swab Sample By RNA:DNA Hybridization In SDIBSS.

An example of the use of the SDIBSS system for rapid detection of Mycoplasma pneumoniae in a clinical sample.

1. (a) Resuspend throat swab material in a suitable solution which is compatible with the bacteria of  
10 interest.

(b) Centrifuge the solution at 13,000 x g for 10 minutes. Discard supernatant.

(c) Resuspend the pellet in 300  $\mu$ l of 400  
W  
(-) SDIBSS containing 3% SDS, 0.03M PB, (PH=6.8),  $10^{-3}$ M EDTA.  
15  $10^{-3}$ M EDTA,  $10^{-3}$ M EGTA and  $I^{125}$  - cDNA complementary to M. pneumonia rRNA.

2. Incubate the mixture at 72°C for one hour and assay for hybridization as described.

This procedure resulted in a rate increase of 100-200  
20 fold over the rate at the reference condition.

EXAMPLE 43

A. Detection Of The Presence Of Mycoplasma In A Tissue  
Culture By Means Of RNA:DNA Hybridization In SDIBSS.

An example of the use of the Sodium Phosphate system for  
25 the rapid detection of Mycoplasma infection of tissue culture is disclosed.

1. (a) Centrifuge 50  $\mu$ l of tissue culture media from a baby hamster kidney cell culture for 5 minutes at 12,000 x g. Discard the supernatant.

(b) Resuspend the pellet in 100  $\mu$ l of 0.15M NaCl.

(c) Mix well:

45  $\mu$ l solution (b).

5 5  $\mu$ l 5% Sarkosyl,  $10^{-2}$ M EDTA,  $10^{-2}$ M EGTA, 0.96M PB, 0.01 micrograms/ml of 3H-cDNA complementary to Mycoplasma hominis rRNA.

65  $\mu$ l 4.8M PB.

2. Incubate at 72°C for one hour and assay for 10 hybridization as described.

This procedure results in a hybridization rate increase of 100-200 fold over the reference condition rate.

#### EXAMPLE 44

B. Detection Of Mycobacteria In A Sputum Sample By  
15 RNA:DNA Hybridization In SDIBSS.

This is an example of the use of the SDIBSS system for the rapid detection of Mycobacteria in a clinical sample.

1. (a) Liquefy sputum and centrifuge solution to pellet Mycobacteria discard supernatant.

20 (b) Suspend pellet in 40  $\mu$ l of 3.3% SDS and add 50  $\mu$ l of glass beads (0.2-0.3mm)(Dyno-Mill brand), sonicate 10 minutes in a mettler M4 ultrasonic cleaner.

(c) Mix well:

Solution from b).

25 100  $\mu$ l 45% (  $\frac{W}{-}$  ) SDIBSS.

176  $\mu$ l 43.5% (  $\frac{W}{-}$  ) SDIBSS, 0.07M PB,  $1^{125}$  -  
cDNA ( $10^{-2}$  micrograms/ml) complementary to Mycobacteria rRNA.

2. Incubate th mixture at 72°C for one hour and assay for hybridization as described.

This procedure resulted in a rate increase of 100-200 fold over the rate at the reference condition.

EXAMPLE 45

C. RNA:DNA Hybridization In A Mixture Of Urea and

5 Sodium Phosphate.

A greatly accelerated rate occurs in a mixture of Sodium Phosphate and Urea.

1. Mix well:

10  $\mu$ l of solution containing 25,000 lysed

10 Legionella bacteria. Total Legionella rRNA equalled  $5 \times 10^4$  micrograms. Solution composition was 1.3% SDS, 0.03M Tris, pH=8.2, 0.37M NaCl  $3.3 \times 10^{-4}$ M EDTA, 0.66 milligrams/ml Proteinase K.

10  $\mu$ l  $H_2O$ .

15 5  $\mu$ l  $I^{125}$  - cDNA complementary to Legionella rRNA.

15  $\mu$ l 10M Urea.

88  $\mu$ l 5M PB.

2. Incubate the mixture at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for 20 hybridization as described earlier.

Little difference in rate or extent of hybridization was seen between this reaction and a control reaction in which water was substituted for the Urea.

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EXAMPLE 46A. Effect of GHCl on RNA:DNA Hybridization in (NH<sub>4</sub>)SO<sub>4</sub>

<u>Concentration of (NH<sub>4</sub>)SO<sub>4</sub> in Reaction</u>	<u>Concentration of of GHCl in Reaction</u>	<u>Relative Extent of Hybridization</u>
2.2M	0	100%
2.2M	1.45M	13%
2.4M	1.28M	35%
2.6M	1.1M	46%
2.75M	1.0M	105%
3.0M	0.8M	96%

B. Effect of GHCl on RNA:DNA Hybridization In Sodium  
Phosphate (PB)

<u>Concentration of (NH<sub>4</sub>)SO<sub>4</sub> in Reaction</u>	<u>Concentration of of GHCl in Reaction</u>	<u>Relative Extent of Hybridization</u>
3.1M	0	100%
3.1M	1.45M	36%
3.3M	1.25M	71%
3.53M	1.1M	90%
3.75M	.92M	95%
3.9M	0.8M	114%

EXAMPLE 47

Detection Of Legionella Bacteria In A Liquid Sample By  
RNA:DNA Hybridization In SDIBSS and Amphyl (Sold By National  
Laboratories). Active Ingredients In Amphyl, 10.5% 0-Phenyl-  
phenol, 5% 0-Benzo-P-Chlorophenol, 84.5% Inert Ingredients.

A greatly accelerated rate occurs in a mixture of Sodium  
Phosphate and Amphyl, a bactericidal agent.

## 1. Mix well:

2  $\mu$ l solution containing  $1.2 \times 10^4$  intact Legionella  
bacteria.

12  $\mu$ l solution containing 4% Amphyl and radioactive  
cDNA complementary to Legionella rRNA.

18  $\mu$ l 4.8M PB.

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2. Incubate at 72°C and at specified times remove aliquots. Dilute each aliquot and assay it for hybridization as described earlier.

This example demonstrates that the rRNA of Legionella bacteria is made available for hybridization by adding Amphyl to the reaction mixture. No pre-cracking of the bacteria is necessary.

In closing, it should be understood that the embodiments of the present invention disclosed herein are illustrative of the principles of the invention and that other modifications, nucleic acid precipitating agents or nucleic acid denaturing agents may be employed which are within the scope of the invention. However, the methods disclosed and described herein are preferred. Accordingly, the present invention is not limited to that precisely as disclosed and described.



CLAIMS

1. An improved method for the formation of double-stranded nucleic acid molecules from separate single-stranded nucleic acid molecules wherein the rate of reaction is greatly increased over the standard reference condition reaction  
5 rate, said method comprising the steps of:

preparing an aqueous reaction solution containing a quantity of a first single-stranded nucleic acid molecule and a quantity of a second single-stranded nucleic acid molecule, said second single-stranded nucleic acid molecule having at  
10 least one segment of base sequences complementary to a corresponding segment of base sequences of said first single-stranded nucleic acid molecule, and a known concentration of at least one nucleic acid precipitating agent, said known concentration being sufficient to greatly accelerate the rate  
15 of reaction over the rate of the standard reference condition reaction;

incubating said aqueous reaction solution at a temperature at which reassociation can occur; and

assaying said incubated aqueous reaction solution for  
20 the presence of double-stranded nucleic acid molecules.

2. A method according to claim 1 wherein said nucleic acid precipitating agent is dihydroxybenzene, detergent, including sodium dodecyl sulfate, sodium diisobutyl sulfosuccinate, sodium tetradecyl sulfate, Sarkosyl, and the  
25 alkali metal salts and ammonium salts of  $\text{SO}_4$ ,  $\text{PO}_4$ ,  $\text{Cl}$ , and  $\text{HCOO}$ .

3. A method according to claim 1 or 2 wherein the known concentration ranges from about 1M to about 10M.
4. A method according to any one of the preceding claims wherein said aqueous reaction solution also contains a known  
5 concentration of a nucleic acid denaturing agent.
5. A method according to claim 4 wherein the known concentration of nucleic acid denaturing agent ranges from about 5% by volume to about 95% by volume.
6. A method according to claim 4 or 5 wherein the  
10 nucleic acid denaturing agent is alcohol ranging in concentration from about 10% by volume to 20% by volume.
7. A method according to any one of the preceding claims wherein the pH of said aqueous reaction solution ranges from about 4 to about 11.
- 15 8. A method according to any one of the preceding claims wherein said temperature ranges from about room temperature to about 90°C.
9. A method according to any one of the preceding claims wherein said nucleic acid precipitating agent is sodium  
20 phosphate,  $\text{NaSO}_4$ ,  $\text{LiCl}$ ,  $(\text{NH}_4)_2\text{SO}_4$ , 13 volume % to 30 volume % Sarkosyl, sodium dodecyl sulfate, sodium diisobutyl sulfosuccinate or sodium tetradecyl sulfate.
10. A method according to any one of the preceding claims wherein the nucleic acid precipitating agent is miscible with  
25 an aqueous solution and capable of precipitating single-stranded nucleic acid molecules from an aqueous solution.

11. A method according to claim 10 wherein the aqueous reaction solution is prepared by mixing an aqueous solution containing the first single-stranded nucleic acid molecule and the second single-stranded nucleic acid molecule with  
5 a second solution containing the nucleic acid precipitating agent.

12. A method according to any one of the preceding claims which includes the step of screening a suspected nucleic acid precipitating agent to determine the effective concentration  
10 of said suspected nucleic acid precipitating agent and to determine the associated incubation temperature necessary to greatly increase the rate of reaction over the standard reference condition reaction rate; and including the nucleic acid precipitating agent in the aqueous reaction solution  
15 at the effective concentration.

13. A method according to claim 12 wherein said suspected nucleic acid precipitating agent is screened through the steps of:

preparing a series of solutions, each of said solutions  
20 containing a known amount of single-stranded nucleic acid and a different amount of said suspected nucleic acid precipitating agent;

examining each of said solutions for the presence of a nucleic acid precipitate; and

25 heating each of said solutions in which a precipitate was found in order to determine whether the degree of

precipitation changes.

14. A method according to any one of the preceding claims used for detecting the presence of bacteria in a sample suspected of containing bacteria, said method comprising:  
5 preparing an aqueous reaction solution containing a quantity of a test sample suspected of containing bacteria, said test sample having been treated with a quantity of denaturing agent sufficient to disassociate the double-stranded nucleic acid molecules present in said bacteria into  
10 single-stranded nucleic acid molecules, a quantity of a second single-stranded nucleic acid molecule complementary to the base sequence of the nucleic acid molecules of the organism to be detected, and a known concentration of at least one nucleic acid precipitating agent, said known concentration  
15 being sufficient to greatly accelerate the rate of reaction over the rate of the standard reference condition reaction; incubating said aqueous reaction solution at a temperature at which reassociation can occur; and assaying said incubated aqueous reaction solution for  
20 the presence of double-stranded nucleic acid molecules of the bacteria to be detected.

15. A method for screening a suspected nucleic acid precipitating agent for use in an accelerated nucleic acid reassociation reaction, said method comprising the steps of:  
25 preparing a series of solutions, each of said solutions containing a known amount of single-stranded nucleic acid

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and a different amount of said suspected nucleic acid precipitating agent;

examining each of said solutions for the presence of a nucleic acid precipitate; and

5 heating each of said solutions in which a precipitate was found in order to determine whether the degree of precipitation changes.



European Patent  
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# EUROPEAN SEARCH REPORT

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Application number

EP 86 30 4429

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A, D	BIOCHEMISTRY, vol. 16, no. 24, 1977, pages 5329-5341, US; D.E. KOHNE et al.: "Room temperature method for increasing the rate of DNA reassociation by many thousandfold: The phenol emulsion reassociation technique" * complete *	1	C 12 Q 1/68
A	US-A-4 533 628 (R. MAAS) * abstract *	1	
A	US-A-4 302 204 (G.M. WAHL) * column 2, lines 40-45 *	1	
A	EP-A-0 133 288 (MILES LABORATORIES) * page 32, paragraph 2 *	1	TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
X	EP-A-0 127 327 (NATIONAL RESEARCH DEVELOPMENT CORP.) * page 3, paragraph 1; examples; page 5, paragraph 2 *	1-5, 7-12	C 12 Q 1/00
The present search report has been drawn up for all claims			
Place of search BERLIN		Date of completion of the search 22-12-1986	Examiner GREEN C.H.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document	